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Modulation of agonist responses at the A_1 adenosine receptor by an irreversible antagonist, receptor–G protein uncoupling and by the G protein activation state

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Abstract

Potency and intrinsic activity of agonists depend on ligand structure, but are also regulated by receptor–G protein stoichiometry. A potential functional reserve in adenosine A_1 receptor-mediated G protein activation was investigated by stimulation of guanosine-5′-(γ-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding by the full agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) and the partial agonist 5′-deoxy-5′-methylthioadenosine (MeSA). Pretreatment of rat brain membranes with the irreversible antagonist 1-propyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]-propyl]-8-cyclopentylxanthine revealed no classical receptor reserve for either agonist. The functional significance of the G protein coupling state of the receptor and occupancy of G proteins by guanine nucleotides was assessed after partial uncoupling of receptor–G protein complexes with *N*-ethylmaleimide and in the presence of increasing GDP concentrations. Agonist EC_{50} values in G protein activation were increased after NEM pretreatment and at higher GDP concentrations, and a decrease in the relative intrinsic activity of MeSA was observed. The shift of agonist concentration–response curves to the right, the decrease in maximal effects and the decrease in relative intrinsic activity of the partial agonist point to a functional reserve which has to be attributed to GDP-free receptor–G protein complexes. The mechanisms of action of FSCPX, NEM and GDP were fully consistent with the two-state model of receptor activation. The apparent reserve revealed by GDP reflects a shift from spontaneously active GDP-free receptor–G protein complexes $(RG)^*$, which can bind [^{35}S]GTPγS, to (RG) occupied by GDP. The abundance of $(RG)^*$ is favored by agonists and by the absence of GDP.

Keywords: Adenosine; Receptor reserve; Two-state model; Potency; Intrinsic activity; Efficacy

1. Introduction

Adenosine A_1 receptors regulate a variety of physiological activities *via* activation of the pertussis-sensitive G proteins G_i or G_o [1,2]. Distinct G protein subunits are involved in the signal transduction mechanisms of A_1 receptors in inhibition of lipolysis [3], in induction of anti-adrenergic effects and stimulation of acetylcholine sensitive K^+ channels in the heart [4] and in the inhibition of L-type Ca^{2+} channels in brain [5]. Although the actions of adenosine through A_1 receptors are well characterized and potentially useful for the development of novel therapeutic agents, no A_1-selective ligands are currently in clinical use, but are presently under study in clinical trials, e.g. in renal failure. Due to the ubiquitous distribution of A_1 receptors, the therapeutically desired effects of even highly selective agonists are confounded by undesired side effects of these agents. For example, A_1 receptor agonists,

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Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate; Cladribine, 2′-deoxy-2-chloroadenosine; DPCPX, 1,3-dipropyl-8-cyclopentyladenosine; FSCPX, 1-propyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]-propyl]-8-cyclopentylxanthine; GTPγS, guanosine-5′-(γ-thio)-triphosphate; MeSA, 5′-deoxy-5′-methylthioadenosine; NEM, *N*-ethylmaleimide; R-PIA, R-N^6-phenylisopropyladenosine.

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when used as anti-arrhythmics, will also affect CNS and renal function and inhibit lipolysis in adipose tissue, since all these tissues express A_1 receptors and are high responsive to A_1 adenosine receptor agonists.

The therapeutic index of receptor agonists could be improved by different efficacies in different tissues. Generally, the effect of a receptor ligand depends in part on the compound, but also on tissue-specific factors, like receptor expression levels or the relative stoichiometry between receptors and G proteins [6,7]. When a receptor couples to multiple G protein subtypes, agonist-mediated trafficking of the stimulus may direct the signal toward a specific subset of G proteins and effectors through induction of multiple distinct conformational states. Agonists may activate all G protein subtypes and effectors coupled to a receptor. Other agonists may preferentially activate one G protein subtype and the consecutive effector system; agonists may differ in their preference for activation of distinct G proteins. It has been realized that this mode of agonist action, which has been termed "signal trafficking," is better described by a three-state model of receptor activation [8] rather than by a simpler two-state model [9], which allows only one activated (R^*) and one inactive (R) receptor state. The existence of multiple active states (R^*, R^{**}, etc.) is supported by the reversal of the rank orders of potency of agonists at a single receptor subtype, e.g. for agonists of the pituitary adenylate cyclase-activating peptide (PACAP) receptor [10] and the *Drosophila* octopamine/tyramine receptor [11].

The three-state model implies that multiple active states of receptors can be induced by different agonists. The reversal of rank orders of potency of agonists has been shown in functional experiments. Different agonists may induce qualitatively distinct effects in response to receptor stimulation, depending on the active conformation of the receptor induced. In the presence of a given agonist, the receptor may be either active or inactive. A receptor can adopt multiple active states (R^*, R^{**}, etc.), which are induced by different agonists, and an inactive state (R). There is no evidence that a single agonist may induce more than one active state of the receptor. For this reason, in binding experiments only two distinct affinity states for agonists were observed.

A major problem of adenosine-related drugs is their action on the cardiovascular system. A_1-induced bradycardia and hypotension preclude the use of A_1 agonists as therapeutic agents, e.g. in hyperlipidemia. Although signal trafficking through A_1 receptors seems impossible [12,13], there is good evidence for improved selectivity of some A_1 receptor partial agonists *in vivo* for antilipolytic effects and greatly reduced hemodynamic effects. We have shown previously that 8-methylamino- and 8-ethylamino-derivatives of N^6-cyclopentyladenosine show substantially higher potency and intrinsic activity in inhibition of lipolysis than in reduction of heart rate [14]. This improved selectivity is probably the result of different tissue sensitivities to A_1 receptor stimulation. The receptor reserve for antilipolytic effects of 8-methylamino- and 8-ethylamino-derivatives of N^6-cyclopentyladenosine was higher than for their bradycardic effects [14].

The relative contribution of receptors, G proteins, G protein activational state and receptor–G protein coupling in determining a functional reserve is not clearly understood. To characterize the relative roles of these factors in the regulation of the sensitivity to A_1 receptor stimulation by full and partial agonists, we have performed an *in vitro* study. The relevance of receptor density was probed by graded inactivation of receptors by the irreversibly acting A_1-selective antagonist 1-propyl-3-[3-[[4-(fluorosulfonyl)-benzoyl]oxy]-propyl]-8-cyclopentylxanthine (FSCPX). The role of A_1 receptor–G protein complexes was determined using uncoupling with the sulfhydryl alkylating agent *N*-ethylmaleimide (NEM). The importance of the guanine nucleotide ligation state of the G protein was investigated in the presence of different GDP concentrations. Adenosine A_1 receptor stimulation after FSCPX or NEM pretreatment and in the presence of different GDP concentrations was assessed by measurement of agonist-induced stimulation of [^{35}S]GTPγS binding to G proteins in rat brain membranes. The degree of a functional reserve was estimated according to the method of Furchgott [15] and Furchgott and Bursztyn [16], as recently described by Morey *et al.* [17]. The effects of reduction of receptor density, receptor–G protein coupling and occupancy of the G protein by guanine nucleotide were evaluated according to the two-state model [9].

2. Materials and methods

2.1. Materials

[^{3}H]DPCPX (120 Ci/mmol), [^{3}H]CCPA (42.8 Ci/mmol), [adenylate-^{32}P]NAD (800 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from New England Nuclear. 1,3-Dipropyl-8-cyclopentyladenosine (DPCPX) was from Research Biochemicals Inc. FSCPX was synthesized in our laboratory. The synthetic procedure which differed from the one described previously [18] has been reported elsewhere [19]. Adenosine deaminase (from calf intestine), 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate (CHAPS), dithiothreitol, GDP and GTPγS came from Roche. CCPA, deoxy-2-chloroadenosine (cladribine), MeSA, NEM, pertussis toxin and bovine serum albumin were purchased from Sigma. Hyperfilm MP was from Amersham. All other chemicals were of the highest purity commercially available and came from standard suppliers.

2.2. Preparation of rat brain membranes

Preparation of membranes from rat forebrain was performed as described [20]. The protein content was deter-

mined according to the method of Peterson [21], using bovine serum albumin as standard.

2.3. Treatment of rat brain membranes with reversible and irreversible antagonists

Membranes (1 mg protein/mL) were incubated in 50 mM Tris–HCl buffer pH 7.4 in the presence of 1 U/mL adenosine deaminase with the indicated concentrations of DPCPX or FSCPX for 30 min at 25°. Membranes were washed nine times through centrifugation (30 min at 100,000 *g* in a Beckman Ti60 rotor at 4°) and resuspended in 50 mM Tris–HCl pH 7.4. The final membrane suspension was frozen in liquid nitrogen and stored at −75° for up to 2 weeks until use.

2.4. Treatment of membranes with NEM

Rat forebrain membranes were incubated at a protein concentration of 1 mg/mL with NEM (1–100 μM) in 50 mM Tris–HCl buffer pH 7.4 for 20 min at 25°. At the end of the reaction period, membranes were centrifuged three times as described above. The incubation was repeated with fresh solutions of NEM, followed by three washes with Tris–HCl buffer (30 min at 39,700 *g* in a Beckman JA17 rotor at 4°). The final pellets were resuspended in the same buffer, frozen in liquid nitrogen and stored at −75° for maximally 2 weeks until use.

2.5. [^{32}P]ADP-ribosylation of membranes by pertussis toxin

Pertussis toxin (200 μg/mL) was preactivated in the presence of 50 mM dithiothreitol for 1 hr at 20°. [^{32}P]ADP-ribosylation was performed for 1 hr at 37° in a total volume of 50 μL containing 100 mM Tris–HCl pH 8.0, 2 mM ATP, 1 mM GTP, 25 mM dithiothreitol, 50 nM [^{32}P]NAD (2 μCi), 50 μg membrane protein and 1 μg activated pertussis toxin per sample. Samples were subjected to SDS–PAGE (10% acrylamide, 15 cm gel length), and the dried gels were subjected to autoradiography.

2.6. [^{35}S]GTPγS binding to rat brain membranes

Binding of [^{35}S]GTPγS to membranes was carried out as described previously [20,22]. Briefly, the incubation medium contained, if not indicated otherwise, 50 mM Tris–HCl pH 7.4, 2 mM triethanolamine, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.5 U/mL adenosine deaminase, 0.2 nM [^{35}S]GTPγS and 0.5% bovine serum albumin. Saturation experiments were performed as isotopic dilution of [^{35}S]GTPγS with unlabeled GTPγS. Agonist-induced increases in GTPγS binding were measured as the difference between basal binding and the binding in the presence of 10 μM CCPA or 100 μM MeSA. Samples (0.5–5 μg of membrane protein) were incubated for 90 min at 25° in a shaking water bath. Non-specific binding was the binding in the presence of 10 μM unlabeled GTPγS. Incubations were terminated by filtration over GF/B glass fiber filters (Whatman), followed by two washes of 4 mL with 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ and 0.02% CHAPS.

2.7. Binding of [^{3}H]DPCPX and [^{3}H]CCPA to rat brain membranes

Membranes (40–100 μg per tube) were incubated in the presence of 0.5 U/mL adenosine deaminase in 500 μL 50 mM Tris–HCl pH 7.4 with 0.02% CHAPS containing 0.3 nM [^{3}H]DPCPX or 0.5 nM [^{3}H]CCPA, if not indicated otherwise, for 3 hr at 25°. Non-specific binding was determined in the presence of 10 μM R-N^6-phenylisopropyladenosine (R-PIA) in [^{3}H]DPCPX binding assays or in the presence of 1 mM theophylline in [^{3}H]CCPA binding experiments. Bound and free radioligand were separated by filtration through GF/B glass fiber filters, followed by two 4 mL washes with ice-cold 50 mM Tris–HCl pH 7.4 containing 0.02% CHAPS.

2.8. Data analysis

K_d, K_H, K_I and K_L values (K_i values for high-, intermediate- and low-affinity states) and B_{max} values in adenosine A_1 receptor binding were calculated by non-linear curve fitting with SCTFIT [23] or GraphPad Prism. Data were fitted to a one-state model if fitting to a two-state model did not improve the fit significantly ($P < 0.05$). Likewise, a two-state model was preferred when fitting to three states did not yield a significantly ($P < 0.05$) better fit. K_d, B_{max} and EC_{50} values for [^{35}S]GTPγS binding were calculated with SigmaPlot. The EC_{50} values and affinity values were determined from three to four independent experiments and are given as geometric means with 95% confidence limits. All other results are arithmetic means ± SEM. Intrinsic activities of the partial agonist MeSA are reported as relative values with reference to the full agonist CCPA set as 100%. Statistical significance of differences was assessed by the Student's *t*-test for paired samples. Multiple comparisons were performed after analysis of variance followed by the multiple comparison Student–Newman–Keuls test. Differences were considered significant when $P < 0.05$.

The method of Furchgott [15] and Furchgott and Bursztyn [16], as recently described by Morey *et al.* [17], was applied to estimate the degree of apparent receptor reserve. The equilibrium dissociation constants (K_A) of CCPA and MeSA and the non-inactivated receptors ($q_{functional}$) as a fraction of the total receptor population were determined by plotting equieffective concentrations of the agonists before [A] and after inactivation [A′]. The fractional receptor occupancy (ρ) at different agonist concentrations was calculated as $\rho = [A]/([A] + K_A)$. As a further estimate of the degree of the reserve, the pharmacological shift

ratio (PSR) was calculated as PSR = K_A/EC_{50}. The intrinsic efficacies (ε) of CCPA and MeSA were compared as $\varepsilon_{CCPA}/\varepsilon_{MeSA} = \rho_{MeSA}/\rho_{CCPA}$.

3. Results

3.1. Effect of FSCPX on ligand binding and G protein activation

FSCPX, but not DPCPX pretreatment of rat forebrain membranes decreased radioligand binding to A_1 adenosine receptors in a concentration-dependent manner (Fig. 1). FSCPX inhibited both agonist ([^{3}H]CCPA) and antagonist ([^{3}H]DPCPX) binding. The extent of inhibition of both radioligands was identical, suggesting that FSCPX did not affect the G protein coupling state of the A_1 receptor.

FSCPX, but not DPCPX pretreatment (0.1, 0.3 or 3 μM each, respectively) decreased A_1 adenosine receptor-stimulated G protein activation, as assessed by stimulation of [^{35}S]GTPγS binding (Fig. 2). None of the antagonists had any influence on basal binding in the absence of agonists. Only FSCPX reduced concentration-dependently the extent of stimulation by the full agonist CCPA or the partial agonists MeSA and cladribine (Fig. 2, left panel). In order to examine if a receptor reserve exists at the receptor–G protein level, concentration–response curves for stimulation of [^{35}S]GTPγS binding by the full agonist CCPA and the partial agonist MeSA were established for control membranes and membranes pretreated with DPCPX or FSCPX, respectively. DPCPX pretreatment changed neither agonist EC_{50} values nor the extent of G protein activation by either agonist (Fig. 3, left panel; Table 1), indicating that the antagonist applied during the pretreatment had been washed out completely. FSCPX pretreatment (0.1–3 μM) reduced the maximum stimulation by agonists of [^{35}S]GTPγS binding (Fig. 3, right panel; Table 1). Agonist EC_{50} values were not affected at FSCPX concentrations of 0.1 or 0.3 μM during the pretreatment.

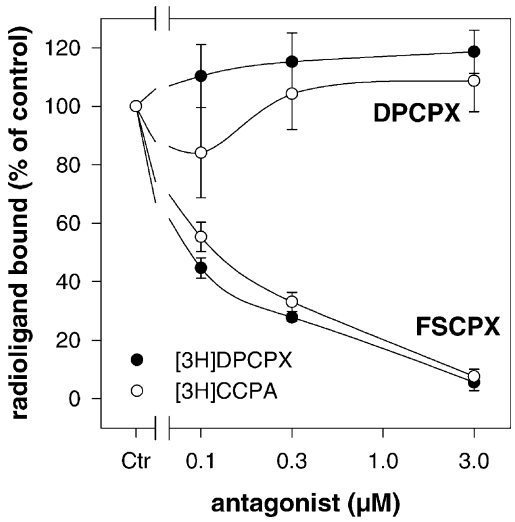


Fig. 1. Effect of antagonist pretreatment on specific radioligand binding to A_1 adenosine receptors. Rat brain membranes (1 mg/mL) were incubated (30 min, 25°) in the absence or presence of DPCPX or FSCPX (0.1–3 μM) and in the presence of adenosine deaminase (1 U/mL). Non-covalently bound antagonist was removed by nine washings. Membranes (40–100 μg per tube) were incubated with [^{3}H]DPCPX (0.3 nM) or [^{3}H]CCPA (0.5 nM), 0.5 U/mL adenosine deaminase in 50 mM Tris–HCl, pH 7.4 containing 0.02% CHAPS for 3 hr at 25°. Non-specific binding was measured in the presence of 10 μM R-PIA ([^{3}H]DPCPX binding) or 1 mM theophylline ([^{3}H]CCPA binding). Control binding was 1521 ± 259 cpm per tube in [^{3}H]DPCPX and 998 ± 245 cpm per tube in [^{3}H]CCPA binding experiments. Data are mean values from four experiments ± SEM.

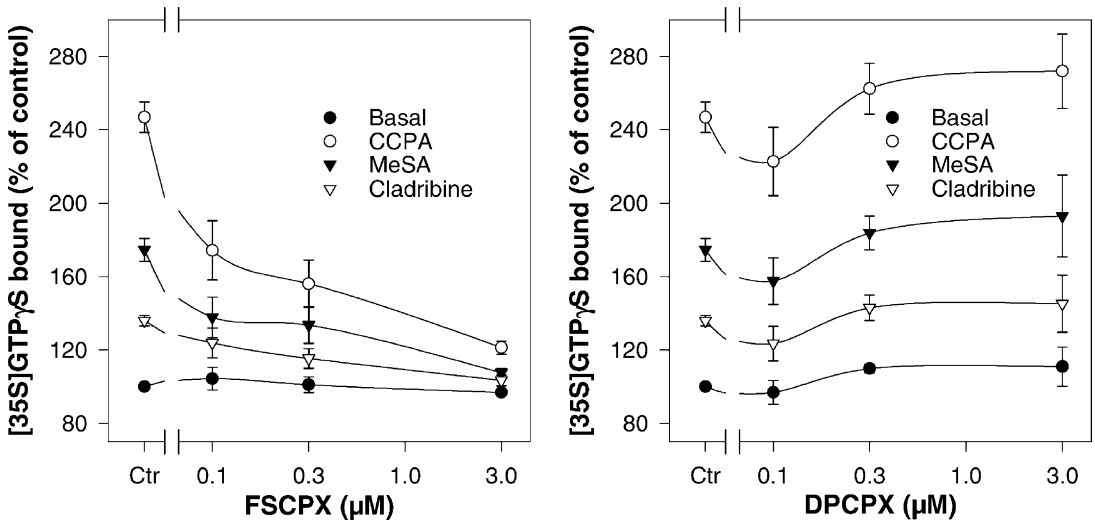


Fig. 2. Effect of antagonist pretreatment on A_1 adenosine receptor-mediated G protein activation. Rat brain membranes had been pretreated with the indicated concentrations of FSCPX (left panel) or DPCPX (right panel). [^{35}S]GTPγS binding to membranes (2 μg per tube) was measured after 90 min incubation at 25° in the absence or presence of 100 μM CCPA, 1 mM MeSA or 1 mM cladribine. Basal binding was 2724 ± 377 cpm per tube in control membranes. Results are means ± SEM from four experiments.

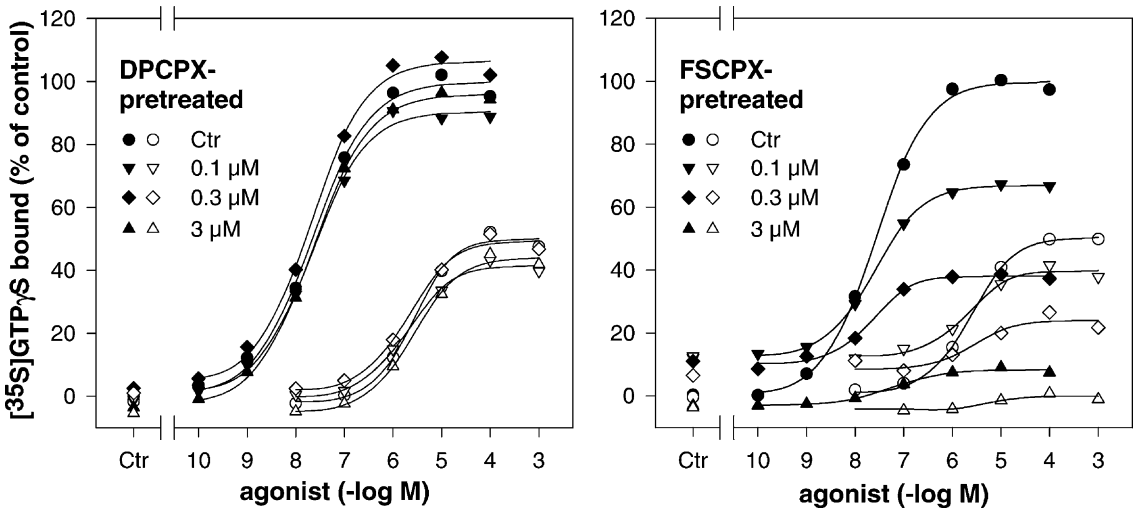


Fig. 3. Effect of antagonist pretreatment on agonist potencies and intrinsic activities in A_1 adenosine receptor-stimulated [^{35}S]GTPγS binding. Stimulation of [^{35}S]GTPγS binding by CCPA (closed symbols) or MeSA (open symbols) to rat brain membranes (2 μg per tube) pretreated without antagonist, DPCPX (left panel; 0.1, 0.3 or 3 μM) or FSCPX (right panel; 0.1, 0.3 or 3 μM) was determined after 90 min incubation at 25°. Data are from one representative experiment out of four experiments.

In membranes pretreated with 3 μM FSCPX, EC_{50} values of CCPA and MeSA were approximately 2-fold higher than in control membranes (Table 1). Pretreatment with 3 μM FSCPX also induced a minor decrease in the relative intrinsic activity of MeSA from 51.3 ± 2.3 to 43.8 ± 3.1% (Table 1). Taken together, these results indicate that at the receptor–G protein level an A_1 adenosine receptor reserve for G protein activation is not detectable by irreversible inactivation of the receptor with FSCPX.

3.2. Effect of receptor–G protein uncoupling with NEM

To consider if a potential functional reserve may consist of receptor–G protein (RG) complexes rather than the receptor alone, A_1 adenosine receptor–G protein complexes were uncoupled by alkylation of the α-subunits of G_i and G_o by pretreatment of the membranes with NEM. This procedure does not affect the A_1 adenosine receptor protein [24]. Because NEM alkylates the same cysteine residue as that affected by pertussis toxin, the effect of the NEM pretreatment can be assessed by pertussis toxin-catalyzed [^{32}P]ADP-ribosylation subsequent to alkylation [25]. In Fig. 4 (upper panel) is shown a progressive decrease in [^{32}P]ADP-ribosylation of G proteins of 40–41 kDa molecular weight when the membranes had been pretreated with increasing NEM concentrations. This indicates that the targets of NEM and pertussis toxin are identical, and that the alkylation by NEM has modified virtually all pertussis toxin-sensitive G proteins. Efficient uncoupling of A_1 receptors from their respective G proteins

Table 1
Potencies and intrinsic activities of CCPA and MeSA in G protein activation in membranes pretreated with DPCPX or FSCPX

Pretreatment	CCPA		MeSA		
	EC_{50} (nM)	Stimulation (cpm)	EC_{50} (nM)	Stimulation (cpm)	RIA (% of CCPA)
Control	20.3 (16.4–25.2)	3721 ± 544	2410 (2090–2790)	2011 ± 290	53.9 ± 0.7
0.1 μM DPCPX	20.8 (17.1–25.2)	3619 ± 410	1990 (1910–2070)	1793 ± 253	49.1 ± 1.8
0.3 μM DPCPX	20.4 (13.6–30.5)	3943 ± 542	2060 (1900–2230)	1987 ± 289	50.2 ± 1.7
3 μM DPCPX	24.3 (19.9–29.7)	3990 ± 675	2250 (2010–2530)	2072 ± 348	49.6 ± 1.4
Control	20.8 (16.8–25.7)	4262 ± 571	2450 (2140–2820)	2207 ± 353	51.3 ± 2.3
0.1 μM FSCPX	20.3 (16.2–25.3)	2141 ± 255	1540 (1280–1840)	1060 ± 129	49.5 ± 0.1
0.3 μM FSCPX	20.1 (13.1–30.8)	1210 ± 98	1440 (935–2210)	661 ± 63	54.4 ± 2.3
3 μM FSCPX	38.8 (24.9–60.6)[a]	678 ± 78	5220 (2980–9160)[b]	296 ± 38	43.8 ± 3.1[c]

[^{35}S]GTPγS binding to rat forebrain membranes (2 μg per tube) was measured in the presence of increasing concentrations of CCPA or MeSA after pretreatment of the membranes with increasing concentrations of DPCPX or FSCPX as outlined in Section 2. The EC_{50} values and the extent of agonist stimulation (basal binding of [^{35}S]GTPγS subtracted from asymptotic maximum) were calculated from concentration–response curves from four experiments. RIA: relative intrinsic activity. Significance of differences was tested with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. control.
[b] Significant difference vs. control, 0.1 and 0.3 μM FSCPX.
[c] Significant difference vs. 0.3 μM FSCPX.

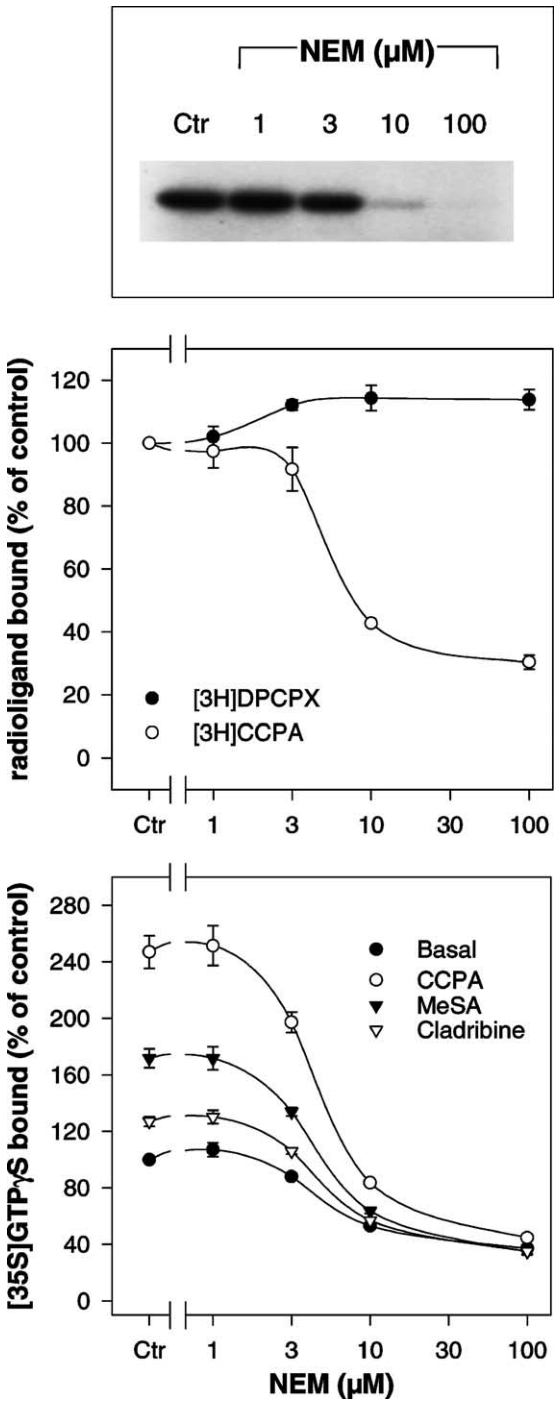


Fig. 4. Influence of pretreatment of rat forebrain membranes with increasing NEM concentrations on the G protein coupling state of the A_1 adenosine receptor. Rat brain membranes (1 mg/mL) were pretreated with or without the indicated concentrations of NEM. Upper panel: the effect of the pretreatment with increasing NEM concentrations was controlled by [^{32}P]ADP-ribosylation of pertussis toxin-sensitive G proteins. Middle panel: [^{3}H]DPCPX (0.3 nM; 40 μg protein per tube) and [^{3}H]CCPA (0.5 nM; 100 μg per tube) binding to A_1 receptors. Control binding was 1740 ± 124 cpm per tube in [^{3}H]DPCPX binding and 651 ± 103 cpm per tube μg for [^{3}H]CCPA experiments. Lower panel: [^{35}S]GTPγS binding in the absence and presence of full (100 μM CCPA) and partial agonists (1 mM MeSA or 1 mM cladribine) after NEM pretreatment. [^{35}S]GTPγS binding to 2 μg membrane protein was measured after 90 min incubation at 25° and amounted to 1797 ± 87 cpm in control membranes. Results are means from four experiments ± SEM.

is indicated by a decrease in A_1 receptor agonist, but not antagonist binding (Fig. 4, middle panel). Binding of [^{3}H]DPCPX was slightly higher in NEM-pretreated membranes than in control membranes. This may be due to the weak inverse agonistic property of DPCPX [26]. Furthermore, increasing NEM concentrations used in the pretreatment also led to lower levels of basal as well as agonist-stimulated [^{35}S]GTPγS binding to G proteins (Fig. 4, lower panel). In addition to this attenuation of the maximal effect of agonists, NEM also had significant effects on the potency of CCPA (Table 2). When NEM concentrations of 10 or 100 μM had been applied in the membrane pretreatment, EC_{50} values of CCPA were 2–3-fold higher than in control membranes. NEM also reduced the relative intrinsic activity of MeSA from 48.4 ± 0.5% in control membranes to 37.0 ± 3.4% at 10 μM NEM. When an NEM concentration of 100 μM had been used for pretreatment, the stimulatory activity of MeSA was completely abolished (Table 2). The results obtained with NEM pretreatment in part support the hypothesis of the existence of a receptor–G protein complex reserve, because NEM in a concentration-dependent manner decreased the potency of the full agonist CCPA and, in addition, reduced the relative intrinsic activity of the partial agonist. Because the uncoupling of RG complexes, but not the irreversible inactivation of the receptor protein with FSCPX revealed some features characteristic of an RG reserve, it has to be concluded that the G protein play a relevant role in a functional reserve at the membrane level.

3.3. Effects of guanine nucleotides

To further probe a potential role of the G protein in an RG reserve, we investigated the functional significance of the guanine nucleotide ligation state of the G protein. In a way similar to NEM, GDP and GTP induced an approximately 50% increase in [^{3}H]DPCPX binding, again pointing to inverse agonistic properties of DPCPX [26], and a decrease in binding of the agonist [^{3}H]CCPA (Fig. 5). In saturation experiments with [^{3}H]DPCPX, GDP concentrations of 10 and 100 μM increased the binding affinity of this antagonist without a change in maximum binding capacity (Table 3). The effect of GDP was further examined in competition experiments of [^{3}H]DPCPX binding by CCPA. GDP shifted the inhibition curves to the right (Fig. 6). Fitting of the competition curves revealed that fitting to three states was significantly better ($P < 0.05$) than a two-state fit for experiments in the absence and presence of 1 μM GDP. A comparison of fitting the data to two or three states is shown in Table 4. CCPA detected three states of high (≈60 pM), intermediate (≈2 nM) and low affinity (≈40 nM). In the presence of 10 and 100 μM GDP, only the intermediate- and low-affinity state were detectable. CCPA binding was shifted by GDP from high- to low-affinity states. The binding affinities to the three states were not altered by GDP (Table 4).

Table 2
Potencies and intrinsic activities of CCPA and MeSA in G protein activation in membranes pretreated with NEM

Pretreatment	CCPA		MeSA		
	EC_{50} (nM)	Stimulation (cpm)	EC_{50} (nM)	Stimulation (cpm)	RIA (% of CCPA)
Control	24.1 (20.4–28.3)	2863 ± 145	2410 (1950–2990)	1387 ± 76	48.4 ± 0.4
1 μM NEM	25.0 (20.2–31.0)	2840 ± 162	2680 (2240–3200)	1378 ± 103	48.6 ± 2.2
3 μM NEM	30.8 (24.0–39.6)	2136 ± 231	2190 (1880–2560)	1004 ± 104	47.1 ± 0.9
10 μM NEM	50.1 (35.8–70.3)[a]	586 ± 73	2250 (1420–3560)	213 ± 23	37.0 ± 3.4[a]
100 μM NEM	75.8 (56.0–103)[a]	203 ± 22	NA	0	0

After pretreatment of rat forebrain membranes with increasing concentrations of NEM, [^{35}S]GTPγS binding to membranes (2 μg per tube) was measured in the presence of increasing concentrations of CCPA or MeSA as outlined in Section 2. Results were calculated from four experiments. NA: not applicable. RIA: relative intrinsic activity. Significance of differences was tested with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. control and all other NEM concentrations.

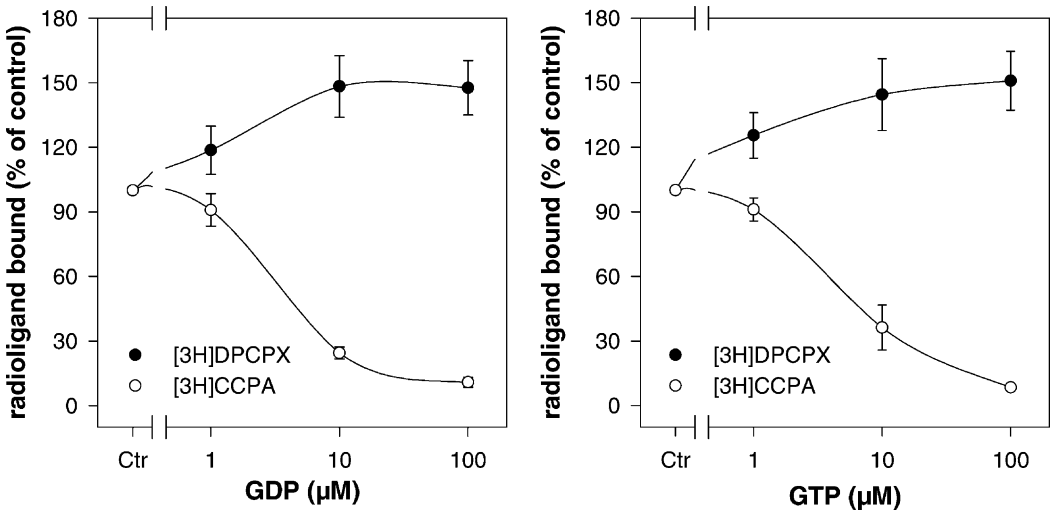


Fig. 5. Effect of increasing guanine nucleotide concentrations on agonist and antagonist radioligand binding to A_1 adenosine receptors. [^{3}H]DPCPX (0.3 nM, 40 μg protein per tube) and [^{3}H]CCPA (0.5 nM, 100 μg protein per tube) binding was performed in the absence or presence of increasing concentrations of GDP (left panel) and GTP (right panel). Specific binding in the absence of guanine nucleotides was 1692 ± 339 cpm per tube in [^{3}H]DPCPX binding and 1075 ± 209 cpm per tube in [^{3}H]CCPA binding experiments. Data are means from three experiments ± SEM.

Saturation of total G proteins with [^{35}S]GTPγS in membranes was performed as isotopic dilution with unlabeled GTPγS in the absence of GDP and agonists. [^{35}S]GTPγS binding amounted to 96,000 ± 10,900 fmol/mg with a K_d value of 1.11 (1.05–1.18) nM (results from three independent experiments; curves not shown). The fraction of A_1 receptor-activated G proteins was assessed

Table 3
Influence of GDP on [^{3}H]DPCPX binding to rat brain membranes

	K_d (nM)	B_{max} (fmol/mg)
Control	0.414 (0.399–0.435)	1217 ± 98
1 μM GDP	0.404 (0.379–0.432)	1292 ± 103
10 μM GDP	0.320 (0.307–0.334)[a]	1282 ± 93
100 μM GDP	0.319 (0.306–0.332)[a]	1252 ± 98

In saturation experiments, 40 μg of rat brain membranes were incubated with increasing concentrations of [^{3}H]DPCPX in the absence or presence of 1, 10 or 100 μM GDP. Results from three independent experiments are shown. Significance of differences was tested with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. control.

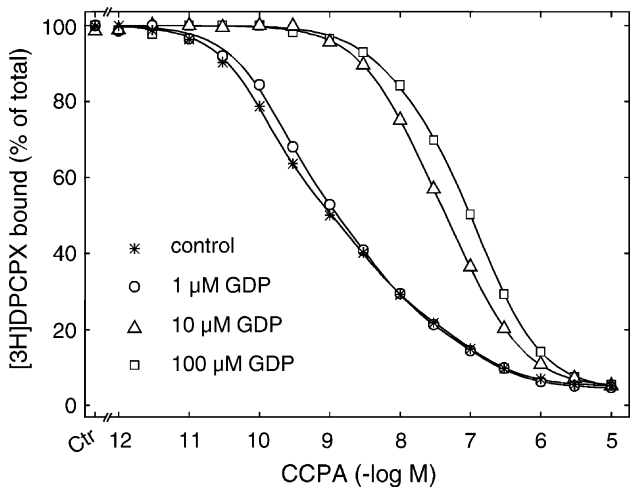


Fig. 6. Inhibition of [^{3}H]DPCPX binding by CCPA in the absence or presence of increasing GDP concentrations. [^{3}H]DPCPX (0.35 nM) was incubated with rat brain membranes (40 μg per tube) in the presence of increasing concentrations of CCPA and in the absence or presence of 1, 10 or 100 μM GDP for 3 hr at 25°. The curves were calculated by fitting the results to a three-state model (control and 1 μM GDP) or to a two-state model (10 and 100 μM GDP), respectively. One out of three experiments is shown.

Table 4
Inhibition of [^{3}H]DPCPX binding by CCPA at different GDP concentrations

	K_H (nM)	% R_H	K_I (nM)	% R_I	K_L (nM)	% R_L
Two states						
Control	0.082 (0.070–0.097)	62.0 ± 1.0			13.5 (10.9–16.9)	38.0 ± 1.0
1 μM GDP	0.099 (0.073–0.135)	62.0 ± 1.6			13.5 (11.0–15.3)	38.0 ± 1.6
10 μM GDP	2.79 (1.11–7.01)[a]	39.7 ± 8.8[a]			36.7 (23.5–57.3)[a]	60.3 ± 8.8[a]
100 μM GDP	1.83 (0.70–4.78)[a]	17.1 ± 4.3[a]			56.2 (44.9–70.5)[a]	82.9 ± 4.3[a]
Three states						
Control	0.052 (0.040–0.068)	50.1 ± 1.3	1.61 (1.46–1.78)	27.7 ± 2.7	43.4 (31.0–60.7)	22.2 ± 2.3
1 μM GDP	0.075 (0.059–0.096)	54.1 ± 1.0	3.57 (2.14–5.95)	31.1 ± 1.2	37.7 (62.9–72.9)	14.8 ± 1.5
10 μM GDP		0	2.79 (1.11–7.01)	39.8 ± 8.8	36.7 (23.5–57.3)	60.2 ± 8.8[a]
100 μM GDP		0	1.83 (0.70–4.78)	17.1 ± 4.3	56.2 (44.9–70.5)	82.9 ± 4.3[a]

Rat brain membranes (40 μg per tube) were incubated in the absence or presence of 1, 10 or 100 μM GDP with 0.35 nM [^{3}H]DPCPX and increasing concentrations of CCPA. The results were fitted to a two-state (upper panel) and a three-state model (lower panel). Fitting the results obtained in the absence and presence of 1 μM GDP to a three-state model improved the fits significantly ($P < 0.05$) compared to a two-state model. The data shown are from three independent experiments. K_H, K_I and K_L denote inhibition constants of CCPA for binding to the high-, intermediate- and low-affinity state. Percent (%) R_H, R_I and R_L are fractions of binding to these states as percentage of specific binding. Significance of differences was tested with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. control.

in the presence of increasing GDP concentrations by saturation of CCPA- and MeSA-induced [^{35}S]GTPγS binding to G proteins (Fig. 7). When the GDP concentration in the [^{35}S]GTPγS binding assay was increased from 1 to 100 μM GDP, the CCPA-induced maximum G protein activation was increased from 5290 to 11,140 fmol/mg (Table 5), consistent with the notion that the receptor acts on G_{GDP} to release GDP from the α-subunit. A_1 receptor-mediated G protein activation in the presence of 10 μM CCPA affected only a fraction of up to 11.6% (11,140 fmol/mg at 100 μM GDP; Table 5) of total G protein (96,000 fmol/mg). From the A_1 receptor saturation data presented in Table 3, it can be deduced that one receptor molecule activates up to ten G protein α-subunits. In contrast to CCPA, the maximum [^{35}S]GTPγS binding capacity in MeSA-induced G protein activation revealed no significant differences at GDP concentrations between 1, 10 and 100 μM GDP (Table 5). Increasing GDP concentrations progressively lowered the apparent affinity of [^{35}S]GTPγS (Table 5), which was as expected because both nucleotides bind to the same site. At the highest GDP concentration, a significantly lower affinity of [^{35}S]GTPγS in MeSA-stimulated binding compared to CCPA-stimulated binding was observed.

The potency and efficacy of CCPA and MeSA were assessed in concentration-dependent stimulation of [^{35}S]GTPγS binding in the presence of 1, 10 or 100 μM GDP, respectively (Fig. 8). The corresponding EC_{50} values and the maximal effects of both agonists are shown in Table 6. In the presence of 1 μM GDP, half-maximal stimulation of [^{35}S]GTPγS binding by CCPA was obtained at a concentration of 4.61 (2.00–10.7) nM. This EC_{50} value is significantly lower than the EC_{50} value of CCPA in the presence of 10 μM GDP (26.7 (20.8–34.2) nM), which, in turn, is also lower than the EC_{50} value measured for this full agonist in the presence of 100 μM GDP (73.9 (68.6–79.5) nM). The extent of stimulation by CCPA (maximum level in the presence of the agonist—non-stimulated binding) was identical in the presence of 1 and 10 μM GDP. In the presence of 100 μM GDP, however, the maximum effect of CCPA was significantly decreased to approximately 40% compared to the level of stimulation when only 1 μM GDP was present (Table 6). The potency of the partial agonist MeSA was affected by GDP in a manner

Table 5
Saturation of CCPA- and MeSA-induced [^{35}S]GTPγS binding to rat brain membranes at increasing GDP concentrations

	CCPA-stimulated		MeSA-stimulated	
	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)
1 μM GDP	1.31 (1.04–1.64)	5290 ± 849	1.53 (1.01–2.32)	4650 ± 1030
10 μM GDP	2.44 (2.08–2.85)[a]	8700 ± 691	3.64 (3.01–4.40)[a]	6170 ± 376
100 μM GDP	8.75 (6.95–11.0)[a]	11140 ± 2210[a]	17.6 (12.0–25.8)[a,b]	6420 ± 1070[b]

Saturation analysis was performed using homologous displacement of [^{35}S]GTPγS with unlabeled GTPγS in the absence or presence of 10 μM CCPA or 100 μM MeSA. The difference between basal and agonist-stimulated [^{35}S]GTPγS binding was fitted to saturation binding isotherms. The results are obtained from three independent experiments. Significance of differences was assessed with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. 1 μM GDP.

[b] Significant difference vs. CCPA-stimulated binding.

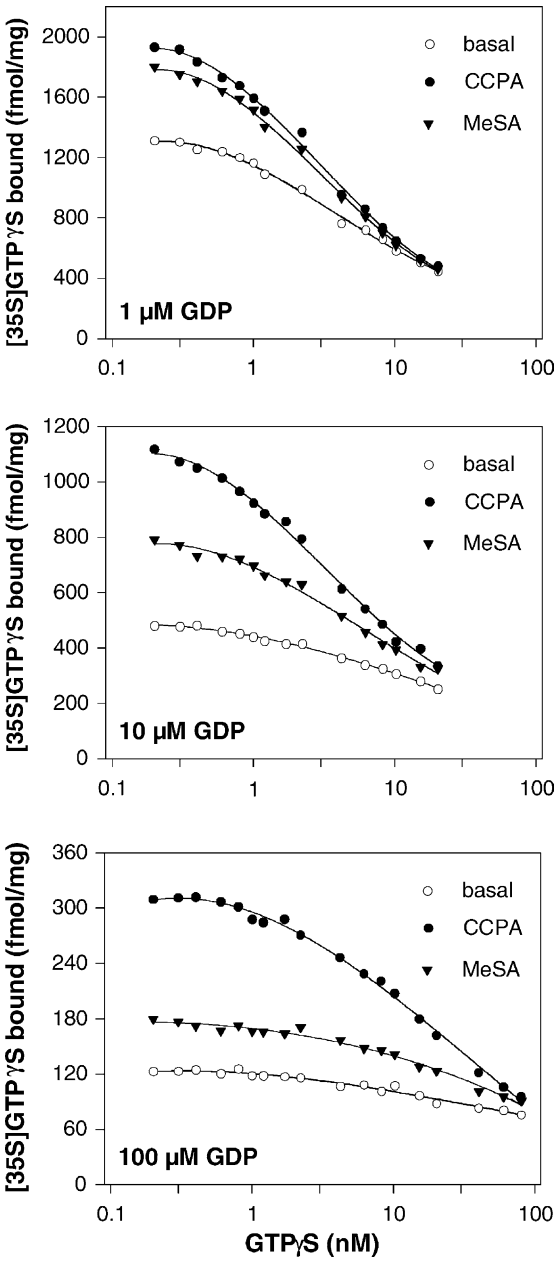


Fig. 7. Saturation analysis of agonist-induced G protein activation in rat brain membranes in the presence of increasing GDP concentrations. Saturation analysis was performed using homologous displacement of [^{35}S]GTPγS with unlabeled GTPγS. The differences between basal and CCPA (10 µM) or MeSA (100 µM) stimulated binding were transformed to obtain saturation binding isotherms, from which K_d and B_{max} values were calculated. The samples contained 2 µg (1 and 10 µM GDP) or 5 µg membrane protein (100 µM GDP). Representative curves from one experiment are shown. Similar results were obtained in two additional experiments.

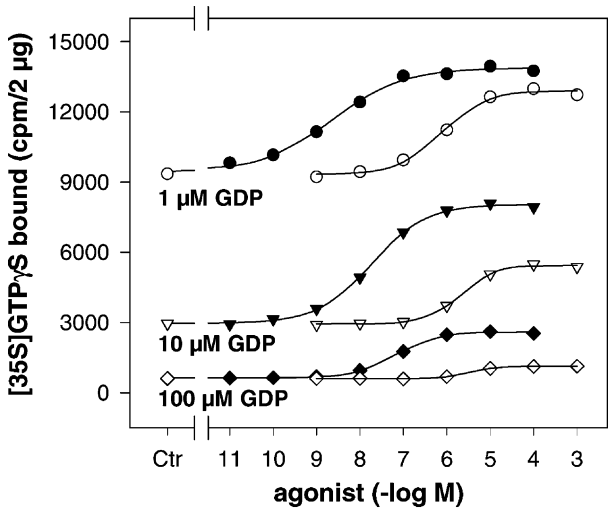


Fig. 8. Concentration–response curves for CCPA and MeSA for G protein activation in the presence of increasing GDP concentrations. [^{35}S]GTPγS binding to 2 µg of rat brain membranes was determined after 90 min incubation at 25° in the presence of the indicated GDP concentrations and in the presence of increasing concentrations of CCPA (closed symbols) and MeSA (open symbols). The figure shows one representative experiment out of four experiments.

similar to that of CCPA. Increasing GDP concentrations led to progressively lower potencies of MeSA. The decrease in potency of MeSA, however, was much smaller than the effect of GDP on EC_{50} values of CCPA. The potency of CCPA at 100 µM GDP was 16-fold lower than at 1 µM GDP, compared to a 4-fold decrease in potency of MeSA. Importantly, also the relative intrinsic activity of this partial agonist was regulated by GDP (Table 6). GDP concentrations from 1 to 100 µM led to a stepwise reduction of the extent of stimulation of [^{35}S]GTPγS binding and to a reduction of the intrinsic activity of MeSA relative to CCPA from 75.6 ± 2.0% (1 µM GDP) to 45.7 ± 1.8% (10 µM GDP) and 25.5 ± 0.8% (100 µM GDP).

The shift of concentration–response curves to the right and the decrease in maximal effects pointed to the presence of a receptor–G protein complex (RG) reserve. Averaged and normalized concentration–response curves for CCPA and MeSA were evaluated to quantify the degree of the reserve. The relationship between equieffective concentrations in the presence of 1 µM GDP [A] and in the presence of 100 µM GDP [A′] for the two agonists is shown in Fig. 9 (mean values from four experiments). The equilibrium dissociation constants (K_A) of CCPA (273.4 nM) and MeSA (5356 nM) were considerably higher than the EC_{50} values (CCPA: 4.61 nM; MeSA: 943 nM) for G protein activation at 1 µM GDP, pointing to an RG reserve for both agonists. The PSR = K_A/EC_{50}, which is depicted by the more rightward position of the concentration–occupancy curves compared to the concentration–response curves (Fig. 10), was 59.3 for CCPA and 5.68 for MeSA, indicating a higher degree of reserve for the full agonist compared to the partial agonist. The ratio of intrinsic efficacies of CCPA and MeSA ($\varepsilon_{CCPA}/\varepsilon_{MeSA}$) was calculated by comparison of fractional receptor occupancies at different agonist concentrations ($\varepsilon_{CCPA}/\varepsilon_{MeSA} = \rho_{MeSA}/\rho_{CCPA}$, with $\rho = [A]/([A] + K_A)$). The relative intrinsic efficacy of CCPA compared to MeSA calculated this way was 9.03. This value is in good agreement with another value for estimation of relative intrinsic efficacies,

Table 6
Potencies and intrinsic activities of CCPA and MeSA in G protein activation in the presence of increasing GDP concentrations

	CCPA		MeSA		
	EC_{50} (nM)	Stimulation (cpm)	EC_{50} (nM)	Stimulation (cpm)	RIA (% of CCPA)
1 μM GDP	4.61 (2.00–10.7)	4412 ± 426	943 (786–1130)	3338 ± 341	75.6 ± 2.0
10 μM GDP	26.7 (20.8–34.2)[a]	4911 ± 628	2550 (2010–3230)[a]	2223 ± 244	45.7 ± 1.8[a]
100 μM GDP	73.9 (68.6–79.5)[b]	1804 ± 256	3850 (3340–4430)[b]	465 ± 104	25.5 ± 0.8[b]

Rat forebrain membranes (2 μg per tube) were incubated with increasing concentrations of CCPA or MeSA in the presence of 1, 10 or 100 μM GDP, respectively. Parameters of concentration–response curves were calculated from four experiments. RIA: relative intrinsic activity. Significance of differences was tested with the Student–Newman–Keuls test for multiple comparisons after analysis of variance.

[a] Significant difference vs. 1 μM GDP.

[b] significant difference vs. 1 and 10 μM GDP.

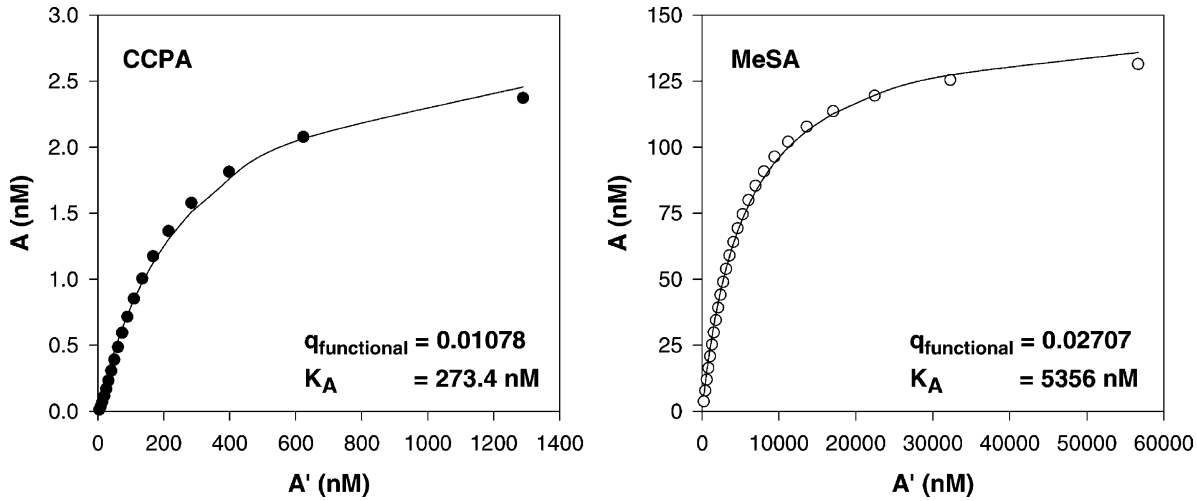


Fig. 9. Comparison of equieffective concentrations of agonists in the presence of 1 and 100 μM GDP. Concentrations of CCPA (left panel) and MeSA (right panel) determined from four independent experiments which caused an equal stimulation (in cpm/μg) above basal levels in [^{35}S]GTPγS binding in the presence of 1 μM GDP (A) and 100 μM GDP (A′) were used to estimate the K_A values and the fraction of non-inactivated receptors ($q_{functional}$).

namely the PSR = K_A/EC_{50}, which was 10.4-fold higher for CCPA compared to MeSA. The relationship between receptor occupancy and the extent of G protein activation is depicted in Fig. 11. This relationship was non-linear for both the full agonist CCPA and the partial agonist MeSA, and pointed to a greater reserve for CCPA. The fractional occupancies for half-maximal G protein activation by CCPA and MeSA were 2 and 30%, respectively.

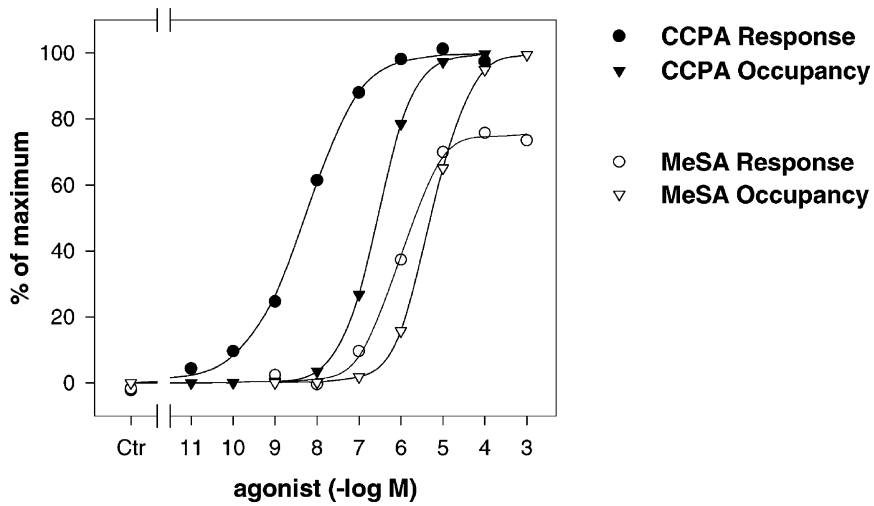


Fig. 10. Receptor occupancy and response in the presence of increasing concentrations of CCPA and MeSA. Receptor occupancy at different agonist concentrations was calculated as $\rho = [A]/([A] + K_A)$. The response curves show normalized averaged data from four experiments of agonist stimulation of [^{35}S]GTPγS binding at 1 μM GDP.

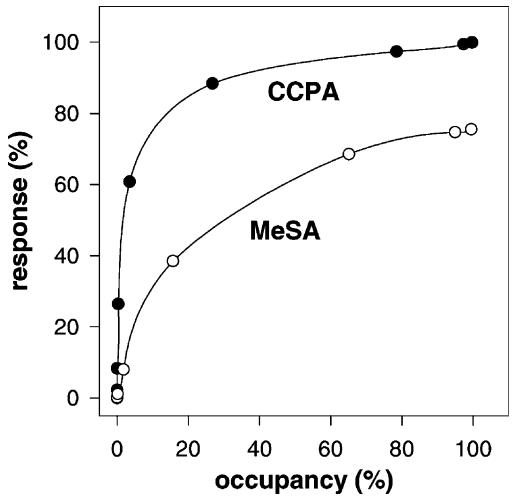


Fig. 11. Relationship between receptor occupancy and the maximum response in G protein activation. The comparison of receptor occupancy by CCPA and MeSA and portion of maximum stimulation of [^{35}S]GTPγS binding at 1 μM GDP was performed using the K_A calculated from the data shown in Fig. 9 and the occupancy calculated as $\rho = [A]/([A] + K_A)$ (Fig. 10). Both curves show a non-linear relationship between receptor occupancy and the induced response.

4. Discussion

The present study characterizes the relative roles of receptors, receptor–G protein complexes and the GDP ligation state of the G protein in determining the extent of A_1 adenosine receptor reserve for G protein activation in rat brain membranes. The presence of a putative receptor reserve in G protein activation has been probed by inactivation of receptors, e.g. by alkylation of the α_{2D}-adrenergic receptor with *N*-ethoxycarbonyl-1,2-dihydroquinoline [27] or by the use of irreversible antagonists, e.g. benextramine for 5-HT_{1A} receptors [28]. The potency and efficacy of agonists in stimulation of [^{35}S]GTPγS binding has also been addressed in cell lines expressing different densities of human 5-HT_{1A} receptors, but identical densities of G proteins [29].

In the present investigation, we have utilized increasing concentrations of the irreversible antagonist FSCPX [18,30] for stepwise inactivation of adenosine A_1 receptors. In agreement with a previous study [30], FSCPX selectively reduced radioligand binding to A_1 receptors without impairment of receptor–G protein coupling, since it decreased binding of the radiolabeled agonist to the same degree as that of the radioligand antagonist (Fig. 1). Graded inactivation of A_1 receptors with FSCPX reduced the extent of G protein activation by CCPA and MeSA. The EC_{50} values of both agonists were identical for stimulation of [^{35}S]GTPγS binding in control membranes and in FSCPX-pretreated membranes (0.1 and 0.3 μM). The relative intrinsic activity of the partial agonist MeSA was unchanged. Based on these results, it must be concluded that in the process of G protein activation by A_1 receptors there is no classical receptor reserve. Similar to the data in the present report, little or no receptor reserve was found for A_1 adenosine receptor occupancy and G protein activation in DDT_1 MF-2 cells, whereas a functional reserve was found at the G protein level [31].

In agreement with the results on A_1 adenosine receptors, Tian *et al.* [27] have determined an absence of a receptor reserve in their study of α_{2D}-receptor–occupancy–response relationship in stimulation of [^{35}S]GTPγS binding to PC 12 cell membranes, using the K_A value for epinephrine from Furchgott analysis. In a study on human α_{2C}-adrenergic receptors expressed in CHO-K1 cells, a 20-fold greater receptor occupancy was required for G protein activation compared to inhibition of cAMP accumulation [32]. In contrast, by using the irreversible antagonist benextramine, an approximately 50% receptor reserve for G protein activation by 5-HT_{1A} receptors stably expressed in HeLa cells has been described [28]. Dopamine induces 50% of maximum [^{35}S]GTPγS binding at an occupancy of only 7% of human dopamine D_3 receptors in CHO cells [33]. Taken together, our results and the findings cited above indicate that the presence of a receptor reserve in the process of G protein activation depends on the system investigated.

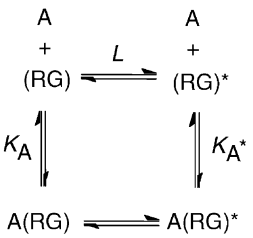
Because inactivation of A_1 receptors with the irreversible antagonist pointed to an absence of receptor reserve, we investigated if a putative reserve might consist of receptor–G protein complexes rather than of the receptor alone. Uncoupling of A_1 receptor–G protein complexes with NEM revealed some features characteristic of a receptor–G protein reserve: EC_{50} values of the full agonist CCPA for G protein activation were higher in membranes which had been pretreated with higher NEM concentrations. In addition, the potency of the partial agonist MeSA was not changed, but the relative intrinsic activity of this compound was lower at 10 μM NEM compared to control membranes and to membranes treated with 1 and 3 μM NEM (Table 2). However, some findings argue against a receptor–G protein reserve revealed by NEM pretreatment: the maximum effect of CCPA on [^{35}S]GTPγS binding was decreased in membranes pretreated with 3 μM NEM without a change in potency; a decrease in potency and maximum effect was found when higher NEM concentrations were applied (Table 2). In conclusion, these results support only partly a receptor–G protein complex reserve. This may be due to "tight coupling" of A_1 adenosine receptors to G proteins, which has been attributed to a protein named the "coupling cofactor," which stabilizes receptor–G protein association [34,35].

As an alternative uncoupling agent, we have used GDP to modulate the quantity of G protein α-subunits available for activation by A_1 receptors. Increasing GDP concentrations favor a holotrimeric GDP-bound, inactive state of G proteins. Increasing GDP concentrations (1, 10 or 100 μM) lead to progressively lower levels of basal [^{35}S]GTPγS binding (Figs. 7 and 8). By adding increasing concentrations of GDP, the percentage of "spontaneously active" G

proteins, which bind [^{35}S]GTPγS without the addition of agonist, is reduced. In the presence of increasing GDP concentrations, the EC_{50} values for G protein activation by the full agonist CCPA and the partial agonist MeSA were increased (Table 6). These results are in accordance with the findings of Pauwels *et al*. [36,37], who described an increase of EC_{50} values for full and partial agonists of human 5-HT_{1A} receptors expressed in C6-glial cells. There are, however, differences regarding the effect of GDP on the relative intrinsic activity of partial agonists. In the present investigation, we have noted a decrease in the relative intrinsic activity of the partial A_1 receptor agonist MeSA. Also for μ-opioid receptors, it has been shown that increasing GDP concentrations decrease the relative intrinsic activity of partial agonists [38]. These findings are in line with the notion that full agonists are better able to overcome the stabilizing effect of GDP on the inactive, holotrimeric G protein. In contrast, the relative intrinsic activity of 5-HT_{1A} and 5-$HT_{1B/D}$ ligands has been shown to be unaffected, decreased or even increased when GDP concentration in the G protein activation assay of human 5-HT_{1A} receptors was increased [36,37]. Our results are in better agreement with the findings of Breivogel *et al*. [39] who found that agonist efficacy at cannabinoid receptors is determined by the decrease in affinity of GDP for G proteins. In agreement with this notion, we have found that the lower the GDP concentration, the more is favored high-affinity agonist binding to A_1 receptors (Figs. 5 and 6, Table 4), and agonist potency and maximum effects are increased (Table 6). This gives rise to an apparent ''receptor'' reserve, which must consist of receptor–G protein complexes devoid of GDP.

The mechanism of action of GDP involves a shift in agonist binding from high- and intermediate-affinity states to low affinity states of the A_1 receptor, but no change in inhibition constants (Table 4). Therefore, the GDP-induced increase in agonist EC_{50} values in G protein activation (Table 6) cannot be attributed to changes in binding affinities, but must be interpreted as a consequence of decreasing the RG reserve at increasing GDP concentrations. The rationale to analyse the results from competition experiments according to a one-, two- or a three-state model was to find the model which best described the experimental data. The simpler model was rejected when the experimental results were better described by a more complex model. In each case, we only used the more complex model when the fit was significantly better than to the simpler model. The finding of three agonist affinity states in inhibition studies of [^{3}H]DPCPX binding in the absence and presence of 1 μM GDP was unexpected, although in some studies [40–42] agonist binding to three distinct states of the A_1 receptor has been reported. We do not assume that three physically distinct binding sites exist on the receptor protein. It should be noted that these sites were detected in binding studies and therefore cannot be directly viewed to represent activational states (active or inactive) of the receptor. In G protein activation studies, the concentration–response curves for CCPA were monophasic. We found no evidence for biphasic responses to CCPA in [^{35}S]GTPγS binding experiments. The high-affinity state found in inhibition of [^{3}H]DPCPX binding is probably not the functionally relevant state for G protein activation by CCPA, because CCPA stimulates [^{35}S]GTPγS binding also in the presence of 10 and 100 μM GDP (Fig. 8, Table 6). At these GDP concentrations, however, the high-affinity state of CCPA binding is not observed (Table 4). The affinity of the intermediate state of CCPA binding, which varies between 1.61 and 3.57 nM (Table 4) is in reasonable agreement with the EC_{50} value of CCPA at 1 μM GDP (4.61 nM; Table 6). The low-affinity binding sites, which predominate at higher GDP concentrations, most probably represent uncoupled receptors. However, the functional role of the three agonist binding sites remains to be elucidated in more detail.

An attempt was made to account for the different effects of FSCPX, NEM and GDP on agonist-induced [^{35}S]GTPγS binding on the basis of a theoretical model. For reasons of parsimony, the system was assumed to behave according to a simple two-state model [9] of receptor activation in which receptors are either inactive (R) or active (R^*). In most studies, R and R^* have not been determined directly. Activation of either G proteins or effectors has served as a more indirect reporting system for receptor activation. Therefore, because we used [^{35}S]GTPγS binding to G proteins as the signal for receptor activation, R and R^* represent the inactive receptor–G protein complex (RG) and the activated, [^{35}S]GTPγS-liganded active complex $(RG)^*$. In the absence of a ligand, the distribution between the two states is determined by the equilibrium constant, L, which is defined by the ratio $[RG]/[(RG)^*]$. In the presence of a ligand (A), which binds to the inactive and active states with dissociation equilibrium constants K_A and K_A^*, respectively, receptors become distributed between four different forms:



From this scheme, the following equation can be derived for the fraction of receptors in the active form [9]:

$$f_{(RG)^*+A(RG)^*} = \frac{[(RG)^*] + [A(RG)^*]}{[(RG)_{TOT}]} = \frac{K_{A^*} + [A]}{K_{A^*}(1+L) + (1 + L(K_{A^*}/K_A))[A]}$$

For the sake of simplicity, it was assumed that [^{35}S]GTPγS activity is directly proportional to the fraction of active

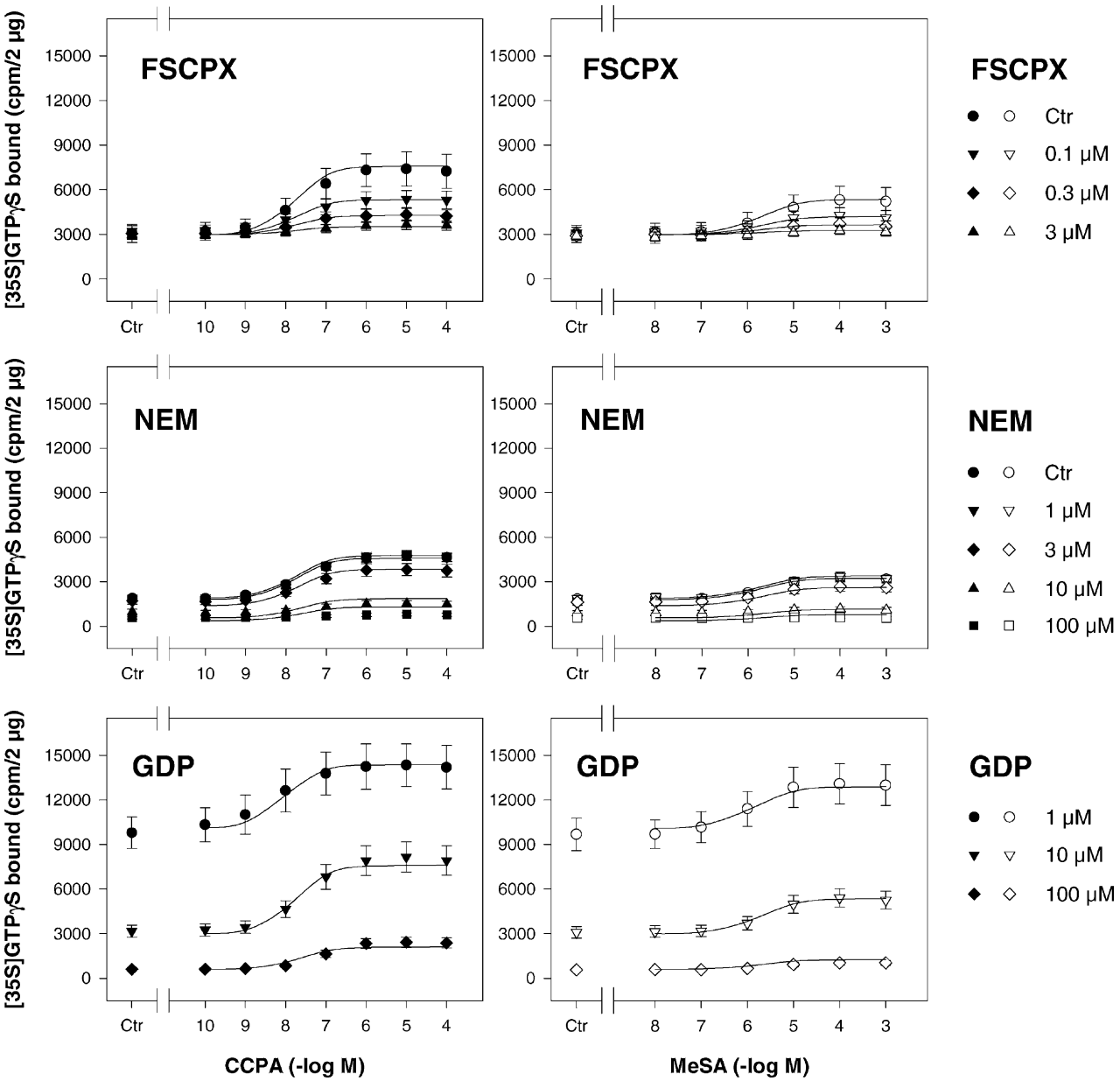


Fig. 12. Simulations by the two-state model of the effects of FSCPX (upper panels), NEM (middle panels) and GDP (lower panels) on CCPA (left panel) and MeSA (right panel) mediated stimulation of [^{35}S]GTPγS binding. The curves superimposed on the mean experimental data points ± SEM were obtained with the equation for $f_{(RG)^*+A(RG)^*+I(RG)^*}$ given in the text which was multiplied by a constant (E_{max}), representing maximal [^{35}S]GTPγS binding. In the simulations, the values of E_{max} (16,948 cpm/2 µg in FSCPX and GDP experiments, 10,252 cpm/2 µg in NEM experiments), K_A (26.65 nM for CCPA, 2214.53 nM for MeSA) and K_{A^*} (6.46 nM for CCPA, 1020.99 nM for MeSA) were fixed. L was varied with concentrations of GDP (0.68, 4.68 and 26.93 for 1, 10 and 100 µM GDP, respectively; lower panels) and concentrations of NEM (4.68, 4.37, 6.34, 17.20 and 26.21 in the presence of 10 µM GDP and after pretreatment without NEM or with 1, 3, 10 and 100 µM NEM, respectively; middle panels). The fraction of inactivated receptors (q) was varied with concentration of FSCPX (0, 0.49, 0.72 and 0.88 for 0, 0.1, 0.3 and 3 µM FSCPX, respectively; upper panels).

receptors. Simulations of experimental data were obtained by multiplying $f_{(RG)^*+A(RG)^*}$ by a constant E_{max}, which represents the maximal possible response. The curves calculated according to the two-state model, which are depicted in Fig. 12, are in excellent agreement with the experimentally obtained [^{35}S]GTPγS binding levels and the concentration–response curves shown in Figs. 3 and 8.

Leff [9] has suggested that the effects of an irreversible antagonist can be modeled by assuming a selective reduction of the concentration of receptors in the activated state. In the present study, however, FSCPX was found to have no effect on basal [^{35}S]GTPγS activity, and agonist potency and the experimental data could therefore not be accounted for by changing the value of L. Instead, it was assumed that FSCPX binds with equal affinity to (RG) and (RG)*, and that following pretreatment, a fraction (q) of the receptors is inactivated and unavailable for binding with the agonist. However, since (RG)* bound to the irreversible antagonist,

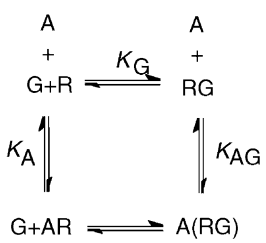
I(RG)*, is still active, no change in basal response will be seen, and the relationship between the fraction of active receptors and agonist concentration is now given by

$$f_{(RG)^*+A(RG)^*+I(RG)^*} = \frac{K_{A^*} + [A]}{K_{A^*}(1+L) + (1+L(K_{A^*}/K_A))[A]}(1-q) + \frac{q}{1+L}$$

Note that when $q = 0$, this equation simplifies to the original equation derived by Leff [9]. Formulated in this manner, the two-state model can account for the observation that FSCPX only reduced maximal agonist response and had no effect on potency and baseline activity. Fig. 12 (upper two panels) shows that adequate simulations of the CCPA and MeSA data was obtained with q values of ~0.5, 0.7 and 0.9 for the 0.1, 0.3 and 3 μM FSCPX treatment groups, respectively. The estimates of the fraction of inactivated receptors are in good agreement with the results of the radioligand binding experiments in which a 55, 72 and 94% reduction in [^{3}H]DPCPX binding was found following incubation with 0.1, 0.3 and 3 μM FSCPX, respectively (Fig. 1).

Because the fraction of (RG)* in the absence of ligand is determined by L, the observation that GDP progressively decreases basal [^{35}S]GTPγS activity can only be explained by the model by assuming a concentration-dependent increase of L by GDP. This is in accordance with the expectations on the effects of GDP from previous work [20,22]. Increasing L will also reduce agonist potency and maximal response, which is consistent with the experimental observations (Fig. 8, Table 6). The simulations of the CCPA and MeSA data in Fig. 12 (lower panels) indicate that in our assay the basal ratio changes from almost 2:1 in favor of (RG)* in the presence of 1 μM GDP to ~5:1 and ~25:1 in favor of (RG) in the presence of 10 and 100 μM GDP. The influence of NEM on receptor activation by CCPA and MeSA is also adequately described by assuming a concentration-dependent increase of L (Fig. 12, middle panels).

The experimental data are equally well described by assuming that R coupled to G is spontaneously active in the absence of agonists. In this case, (RG) and (RG)* are not discriminated, and K_{A^*} and L are rephrased to K_{AG} and K_G/[G]. The corresponding model is



Based on this model, the fraction of active receptor–G protein complexes is given by

$$f_{RG+A(RG)} = \frac{K_{AG} + [A]}{K_{AG}(1+(K_G/[G])) + (1+(K_{AG}K_G/K_A[G]))[A]}$$

It should be noted that this model and equation are mathematically identical to those developed by Leff [9].

In summary, we have shown that agonist responses in G protein activation by A_1 adenosine receptors are affected by FSCPX, NEM and GDP in a different manner. These differences are consistent with a two-state model. The FSCPX experiments demonstrate that there is no "classical" receptor reserve in native brain membranes. In this system, in which a classical receptor reserve is absent, a reserve still exists such that maximal responses can occur when the capacity of the receptor to couple to and activate G proteins is reduced. The apparent reserve revealed by GDP, which consists of GDP-free receptor–G protein complexes, is a direct consequence of the behavior of the two-state model and reflects a shift from (RG)* to (RG) in the system. The abundance of (RG)* is favored by agonists and by the absence of GDP. A spare receptor–G protein complex is in the GDP-free form.

Intracellular GDP concentrations are around 50 μM. Even at 100 μM GDP, we found that 17% of A_1 receptors were in the high-affinity state (Table 4). High GDP concentrations reduced basal [^{35}S]GTPγS binding, but increased the B_{max} of CCPA-stimulated [^{35}S]GTPγS binding (Table 5). We deduce that the function of high intracellular GDP is to reduce "noise" and to augment agonist-stimulated G protein activation. In living cells, we may not expect significant changes in GDP concentrations to occur. However, in different tissues with distinct densities of receptor and G protein, intracellular GDP will determine the RG/RG* ratio. GDP will occupy a constant percentage of G proteins. The higher the density of G proteins, the higher will be the absolute number of GDP-free G proteins per cell, which may interact with a receptor and be spontaneously active.

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